Appendix 4

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Monoallelically Expressed Gene Related to p53 at 1p36, a Region Frequently Deleted in Neuroblastoma and Other Human Cancers

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Summary

We describe a gene encoding p73, a protein that shares considerable homology with the tumor suppressor p53. p73 maps to 1p36, a region frequently deleted in neuroblastoma and other tumors and thought to contain multiple tumor suppressor genes. Our analysis of neuroblastoma cell lines with 1p and *p73* loss of heterozygosity failed to detect coding sequence mutations in remaining *p73* alleles. However, the demonstration that *p73* is monoallelically expressed supports the notion that it is a candidate gene in neuroblastoma. p73 also has the potential to activate p53 target genes and to interact with p53. We propose that the disregulation of p73 contributes to tumorigenesis and that p53-related proteins operate in a network of developmental and cell cycle controls.

Introduction

Spontaneous lesions in the gene encoding the tumor suppressor p53 have been implicated in the progression of a wide range of human tumors (Nigro et al., 1989; Hollstein et al., 1991; Levine et al., 1995). The prevalence of tumors in individuals or mice bearing constitutional *p53* mutations suggests that loss of p53 activity also contributes to the generation of tumors (Li and Fraumeni, 1969; Malkin et al., 1990; Donehower et al., 1992; Jacks et al., 1994). Wild-type p53 can also be neutralized through direct interaction with either cellular proteins or viral tumor antigens (Lane and Crawford, 1979; Linzer and Levine, 1979; Werness et al., 1990). p53 appears to induce cell cycle arrest or apoptosis in response to cellular stresses such as DNA damage and hypoxia (Kastan et al., 1992; Livingstone et al., 1992; Lowe et al., 1993; Hartwell and Kastan, 1994). By this means, p53 acts as a tumor suppressor; its loss of function appears to confer selective advantages on cells through deregulated growth and resistance to cell death (Graeber et al., 1996; Kinzler and Vogelstein, 1996).

Despite the widespread presence of *p53* mutations in human malignancies, many tumors develop in the absence of *p53* abnormalities or obvious tumor antigens, most likely due to a loss of other tumor suppressor genes (Weinberg, 1993). Many of these tumor suppressor genes, including *Rb* in retinoblastoma, *NF1* in neurofibramatosis, and *DCC* and *APC* in colon carcinomas, were initially identified through cytogenetic evidence of loss of heterozygosity (LOH) (Benedict et al., 1983; Cavenee et al., 1983; Ballester et al., 1990; Buchberg et al., 1990; Fearon et al., 1987).

Similarly, extensive investigations of neuroectodermal tumors, including neuroblastoma, melanoma, and multiple endocrine neoplasm, have suggested the presence of multiple tumor suppressors at the subtelomeric region of chromosome 1 (Brodeur et al., 1977; Balaban et al., 1986; Ross et al., 1995; Sozzi et al., 1988; Dracopoli et al., 1989). Neuroblastomas with 1p LOH can be subdivided into two classes as having either small deletions (5 to 10 Mb) at 1p36.2-3 or a clinically more aggressive form characterized by *N-myc* amplification and larger deletions of chromosome 1 including subbands p36 and p35 (Brodeur et al., 1984; Takeda et al., 1994; Caron et al., 1995). Notably, the chromosome that sustains the discrete 1p36 deletion of the first class of neuroblastoma is almost exclusively of maternal origin, indicating that the putative tumor suppressor in this region is imprinted (Barlow, 1995; Caron et al., 1995). In contrast, *N-myc*-amplified neuroblastomas show a LOH at 1p35-1p*ter* from either chromosome (Caron et al., 1995; Cheng et al., 1995). These observations suggest that neuroblastoma develops through different mechanisms of inactivating alleles of putative tumor suppressors at 1p36 and that additional genes, at 1p35 and at other loci, influence tumorigenesis. The etiology of neuroblastoma is complicated by an additional class of neuroblastoma, referred to as 4S, that initially appears as a widely disseminated, aggressive disease (Ambros et al., 1995). Remarkably, a majority of the 4S neuroblastomas suddenly and spontaneously regress in the absence of treatment. Understanding these complex pathways of neuroblastoma induction and progression will require the identification of the provisional tumor suppressors located on the short arm of chromosome 1.

We have identified a novel gene encoding a protein, termed p73, with remarkable sequence similarity to the DNA-binding, transactivation, and oligomerization domains of p53. We show that p73 has oligomerization and transactivation properties similar to p53 and that the *p73* gene maps to the 1p36.33 region frequently deleted in neuroblastoma and other tumors. In addition, we provide evidence to support the notion that alterations in *p73* gene expression may be one factor in the development of neuroblastoma and other tumors.

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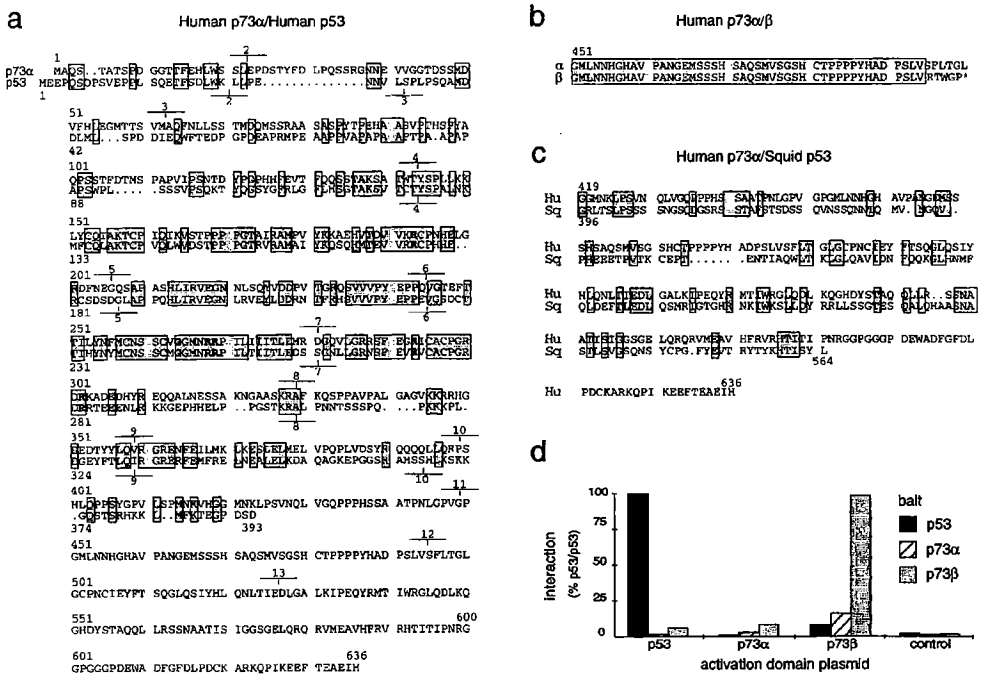


Figure 1. Homology between p73 and p53

Primary amino acid sequences of human p73α and β were deduced from cDNAs from normal human tissues.

(A) Comparative alignments of coding sequence and gene structure of human p73α and p53. Identities are enclosed in shaded boxes, with p53 residues frequently mutated in tumors presented in bold. The identity and position of introns within *p73* and *p53* genes are denoted above and below the respective sequences.

(B) C-terminal sequence of human p73β, a splicing variant lacking exon 13, is aligned with its site of divergence from p73α.

(C) Homology between the C-terminal sequence of p73α and that of a p53-like protein from squid.

(D) Summary of yeast two-hybrid interaction assays between p53, p73α, and p73β, presented as ordinate values relative to B-galactosidase activity of p53–p53 interactions.

Results

Homology between p73 and the p53 Tumor Suppressor

A cDNA encoding p73 was fortuitously discovered in a hybridization screen of a COS cell cDNA library using degenerate oligonucleotides corresponding to IRS-1-binding domains. The coding sequence of *p73* was found to lack any homology to IRS-1 binding domains. Subsequently, libraries of normal human colon tissue cDNAs were screened by hybridization to yield cDNAs encoding p73α and p73β, which are splicing variants of *p73* differing at their C termini (Figures 1a and 1b). The homology between p73 and p53 is extensive within the most conserved p53 domains (Zambetti and Levine, 1993; Ko and Prives, 1996) involved with transactivation (29% identity with p53 amino acids 1–45), DNA binding (63% identity with p53 amino acids 113–290), and p53 oligomerization (38% identity in p53 sequence from 319–363) (Figures 1a and 1c). While the homology between the N terminus of p73 and that required for transcriptional activation by p53 is not strong, a sequence similar to the MDM2-binding domain of p53 (TFSDLW; Lin et al., 1994a) is present in p73 as TFEDLW. Significantly, residues corresponding to those of p53 frequently mutated in tumors (R175, G245, R248, R249, R273, and R282) and shown to be required for sequence-specific DNA recognition (Lin et al., 1994b; Ko and Prives, 1996) are conserved and occupy identical positions in p73 (Figure 1a). No significant homology was detected between the C-terminal domain (364–393) of mammalian p53 and p73. However, the C-terminal domain of human p73α shows homology with recently discovered invertebrate p53 homologs (Figure 1c), suggesting the possibility that *p53* may have evolved from a more primitive, *p73*-like gene. In support of this concept, the intron–exon organization of the *p73* gene was found to be similar to that of the *p53* gene (Figure 1a).

p73β is encoded by transcripts lacking the 96 nucleotides corresponding to exon 13. This deletion interrupts the open reading frame, yielding a polypeptide of 499 amino acids (Figure 1b). Both p73α and β transcripts were detected by PCR in all human tissues tested, including brain, kidney, placenta, colon, heart, liver, spleen, and skeletal muscle (data not shown), indicating a widespread, albeit low level, expression of these proteins.

Considering the extensive homology between p53 and p73, including that in the oligomerization domain of p53, we assessed whether these proteins would interact in the context of the yeast two-hybrid system (Gyuris et al., 1993). Using this assay, we detected strong homotypic interactions between p53 molecules, indicative of their known ability to form oligomers (Ko and Prives, 1996). In contrast, p73α showed a very low tendency to form homotypic interactions in this assay. However,

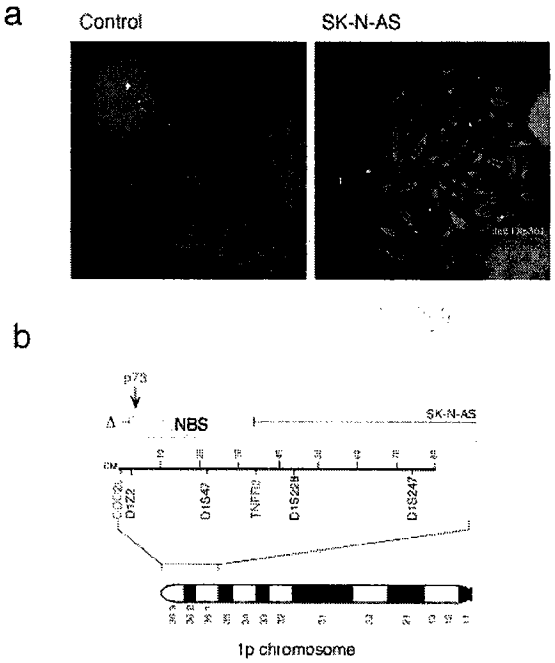


Figure 2. Mapping of *p73* Gene to 1p36 and LOH in Neuroblastoma Cell Line

(A) (Left panel) Fluorescence in situ hybridization (FISH) of a cosmid probe containing the *p73* gene on a normal human chromosome spread. Chromosomes (in red) are counterstained with propidium iodide, and *p73* gene signals appear as green at the telomere of 1p. (Right panel) FISH analysis on chromosome spreads of the SK-N-AS neuroblastoma cell line reveals two chromosome 1 homologs using the centromeric D1Z5 probe (red) but only one chromosome 1 with a p73 signal (green). Chromosomes are counterstained with DAPI (blue).

(B) Summary of cytogenetic data for the localization of the neuroblastoma suppressor locus at 1p36, based on overlapping regions of deletion in neuroblastoma cell lines and detailed mapping of the 1p deletion in the SK-N-AS cell line (Cheng et al., 1996). Approximate positions of genes (in red) and polymorphic markers (in black) are noted.

p73β displayed strong homotypic interactions, equivalent to that of p53. The potential for heterotypic interactions was also assayed by the two-hybrid system. Interestingly, p53 and p73β displayed significant mutual interactions in either bait or prey configuration, while p53 showed negligible interactions with p73α. Weak but detectable interactions between p73α and p73β were also evident in this assay. The physiological significance of the apparent oligomerization and restricted heterotypic interactions amongst p73α, p73β, and p53 determined in these assays remains to be established.

p73 Gene Localized to Neuroblastoma Suppressor Locus at 1p36

To test the possibility that the *p73* gene was located at sites of suspected tumor suppressor genes, we mapped the *p73* gene using fluorescence in situ hybridization (FISH) on normal human chromosome spreads. The p73 cosmid probe hybridized to the subtelomeric p36 region of chromosome 1 (Figure 2a). As deletions in the short arm of chromosome 1 appear frequently in neuroblastoma (Takeda et al., 1994; Caron et al., 1995; Cheng et al., 1995), we performed *p73* FISH analysis on various human cell lines established from neuroblastoma tumors. The SK-N-AS has the smallest 1p deletion of defined neuroblastoma cell lines covering approximately 8 Mb, limited distally by D1Z2 (1p36.33) and proximally by *TNFR2* (1p36.2) (Figure 2b; White et al., 1995; Cheng et al., 1996). Probing of SK-N-AS with the *p73* gene yielded signal from only one of the two chromosome 1 homologs, demonstrating a *p73* LOH in this neuroblastoma cell line (Figure 2a). Subsequent, more detailed mapping has indicated that the *p73* gene is very close to the D1Z2 marker at 1p36.33, thereby placing *p73* at the distal border of the consensus region of deletion in neuroblastoma (Figure 2b).

A similar FISH analysis of neuroblastoma cell lines showing *N-Myc* amplification and a larger 1p deletion, including IMR-32 and CHP-212, confirmed the LOH of the *p73* gene in these cells (Table 1). Interestingly, both the SK-N-SH and the SK-N-MC neuroblastoma cell lines show neither 1p (Davidoff et al., 1992; Cheng et al., 1996) nor *p73* LOH (Table 1), indicating that either other lesions are responsible for these tumors or that *p73* is inactivated by mechanisms besides deletions in these cell lines.

p73 Expression in Neuroblastoma Cell Lines

To examine *p73* and *p53* expression in neuroblastoma cell lines, we developed sensitive RT-PCR reactions to yield amplicons corresponding to the entire coding sequences of *p73* and *p53* transcripts. RT-PCR products of *p73* transcripts were first obtained from IMR-32, SK-N-MC, SK-N-SH neuroblastoma cell lines, as well as the HT-29 colon carcinoma cell line, but not from the SK-N-AS cell line (Figure 3a). To gain quantitative information on levels of *p73* transcript expression, Northern analysis was performed on these same cell lines. Two *p73* transcripts of 4.4 and 2.9 kb, corresponding to polyadenylation variants as determined by direct sequencing, were detected in HT-29, IMR-32, and SK-N-SH mRNA, while *p73* transcripts were either not detected in RNA from SK-N-AS cells or present at exceeding low levels in SK-N-MC cells (Figure 3b, upper panel). Corresponding Northern analysis of *p53* transcripts in these cell lines revealed a common 2.5 kb transcript except for SK-N-MC, which displays a previously characterized truncation (Figure 3b, lower panel; Davidoff et al., 1992). Western blots to detect the p73α protein were performed using a polyclonal antibody directed against the C terminus of p73α. Reflecting the Northern data for *p73* transcripts in these cell lines, extracts from HT-29, IMR-32, and SK-N-SH cells showed easily detectable levels of the p73α protein. However, SK-N-MC and SK-N-AS extracts contained significantly reduced levels of p73α, showing only a faint band that migrates somewhat faster than the main product seen for HT-29, IMR-32, and SK-N-SH (Figure 3c). To characterize further the expression pattern of *p73*, we performed immunolocalization of the endogenous p73α in an array of cell lines, all of which revealed a pattern of small, punctate dots in the nucleus of some cells in an asynchronous

Table 1. Analysis of p73 in Neuroblastoma and Other Tumor Cell Lines

Cell Lines	1pΔ[1]	Gene Copy #[2]	D1Z5 Copy #[3]	Allele(s)[4]	Expressed Allele[5]	Transcript Sequence[6]	Protein[7]	p53 Transcript[8]
Neuroblastomas								
(N-Myc amplified)								
IMR-32	Yes	1	3	A/T; (−)	A/T	wt	+	wt
CHP-212	Yes	1	3	A/T; (−)	A/T*	wt	−	wt
SMS-BC	Yes	NT	NT	G/C; (−)	G/C*	wt	−	mut [135C→F]
SK-N-BE(2)	Yes	NT	NT	G/C; (−)	G/C	wt	−	mut [135C→F]
SMS-KAN	Yes	NT	NT	G/C; (−)	G/C*	wt	−	wt
(N-Myc single copy)								
SK-N-AS	Yes	1	2	G/C; (−)	G/C*	wt	−	wt
SK-N-MC	No	2	2	G/C;G/C	G/C	wt/mut [472A→T]	−	Δexons2–4
SK-N-SH	No	2	2	G/C;A/T	A/T	wt/Δexon2[δ]	+	wt
Others								
NCI-1011	NR	NT	NT	G/C; (?)	G/C	wt	+	mut [176C→F]
HT-29	NR	NT	NT	G/C; (?)	G/C	wt	+	mut [273R→H]
A-172	NR	NT	NT	G/C; (?)	G/C	wt	+	wt
U-373	NR	NT	NT	G/C; (?)	G/C	wt	+	mut [273R→H]
K-562	NR	NT	NT	G/C; (?)	G/C	wt	+	frame shift
IM-9	NR	NT	NT	G/C; (?)	G/C	wt	+	wt
SAOS-2	NR	3	5	G/C;G/C	G/C	wt	−	(-)
MCF-7	NR	3	2	G/C;G/C	G/C	wt	+	wt
U-937	NR	3	4	G/C;G/C	(-)	N/A	−	(-)
HL-60	NR	2	2	G/C;G/C	(-)	N/A	−	(-)
SW-480	NR	NT	NT	G/C; (?)	G/C	wt	+	mut [273R→H] mut [309P→S]

[1]1pΔ, deletion in short arm of chromosome 1, as cited in text. NR, none reported.
[2]Determined by fluorescence in situ hybridization (FISH) with a p73 genomic probe on chromosome spreads, performed as described in Experimental Procedures. NT, not tested.
[3]Determined by FISH analysis with the chromosome 1 centromeric probe, D1Z5 (Oncor). NT, not tested.
[4]Refers to the polymorphism of G/C and A/T alleles, as determined by PCR on genomic DNA and PCR-RL (see Figure 4 and Experimental Procedures) of products. Minus sign (−) indicates *p73* loss of heterozygosity, assumed on the basis of known 1p36 deletions. For cell lines in which chromosome 1p deletions have not been reported (NR), the A/T allele was never detected, though the presence of other non-G/C or A/T alleles cannot be formally excluded (denoted by question mark).
[5]Determined by RT-PCR on mRNA and subsequent sequencing of products. Asterisks indicate a second, 30-cycle round of PCR was required to generate products for sequencing. (-) indicate a lack of detectable product, despite two rounds of RT-PCR.
[6]Determined by sequence analysis of open reading frame in expressed transcripts. wt, wild type compared with normal human tissue (not shown); N/A, not available due to lack of mRNA expression, as determined by RT-PCR. δ indicates 10% of transcripts sequenced contained a deletion in exon 2.
[7]Detectable by Western blotting of whole cell lysates, as described in Figure 3 and Experimental Procedures.
[8]Same as 6, performed on *p53* mRNA.

population (Figure 3d). A similar distribution of dots was revealed upon transfecting mammalian cells with a myc epitope-tagged p73α expression vector, indicating that p73 autonomously targets to these intranuclear foci (Figure 3d).

Lack of Coding Region Mutations in *p73* Gene

As most neuroblastoma cell lines examined displayed a LOH of the *p73* gene, we asked whether the remaining allele sustained genetic changes that might affect *p73* function. We used the p73 RT-PCR reaction to amplify the coding region of p73 from mRNA of an extended group of neuroblastoma cell lines. As with SK-N-AS, the neuroblastoma lines SMS-BC, CHP-212, and SMS-KAN showed no RT-PCR product corresponding to p73 after 30 cycles, suggesting very low expression levels. However, reamplification for an additional 30 cycles yielded sufficient product for sequencing p73 transcripts from these lines. These PCR products, including six from neuroblastoma cell lines displaying a 1p36 LOH (IMR-32, CHP-212, SMS-BC, SMS-KAN, SK-N-BE(2), and SK-N-AS), two from neuroblastoma cell lines that showed no 1p deletion (SK-N-SH and SK-N-MC), as well as those from control, nonneuroblastoma lines, were sequenced in their entirety and found to be identical to *p73* sequences derived from normal human tissue (Table 1). Western blot analysis of the four neuroblastoma lines that showed extremely low levels of p73 transcript by RT-PCR revealed a corresponding lack of p73α protein (Table 1). In addition, two neuroblastoma lines, SK-N-BE(2) and SK-N-MC, showed detectable levels of p73 transcripts by one round of PCR but no p73α protein. These results, together with the absence of mutations in the remaining allele of *p73* LOH neuroblastoma cell lines, suggest that epigenetic mechanisms such as imprinting and translational suppression act to limit *p73* expression in neuroblastoma cells.

Monoallelic Expression of the *p73* Gene

Through our analysis of p73 transcripts in neuroblastoma cell lines and tumors, we discovered an allelic polymorphism consisting of a double nucleotide substitution (G→A) and (C→T) at positions 4 and 14 of exon 2, just upstream of the initial AUG of p73 (Figure 4a). We denote these two naturally occurring *p73* alleles G/C and A/T. The G/C, A/T polymorphism occurs in a region

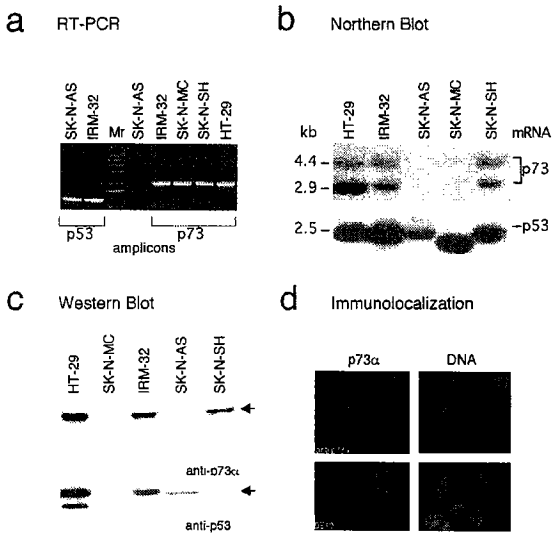


Figure 3. Analysis of *p73* Expression in Cell Lines

(A) RT-PCR products corresponding to the coding regions of p53 and p73α from various cell lines used for sequence analysis (see text).

(B) Northern blot analysis of p73 and p53 transcripts in selected cell lines. The 4.4 and 2.9 kb p73 transcripts result from the use of distinct polyadenylation sites. All cell lines show a similar 2.5 kb p53 transcript except SK-N-MC, which contains a previously characterized deletion (Davidoff et al., 1992).

(C) Western blot analysis of total cell lysates cell lines using polyclonal antibody directed to C terminus of p73α (top) and monoclonal antibody to p53 (bottom).

(D) Immunolocalization of endogenous and transfected p73α in cell lines. (Top left panel) Immunolocalization of endogenous p73α in U251 human glioblastoma cell line using anti-p73α antibodies, revealing numerous discrete foci with the nucleus of some, but not all cells in field. (Top right panel) Hoechst dye staining of field corresponding to left panel, revealing nuclei of cells. (Bottom left panel) Immunolocalization of myc epitope-tagged p73α in baby hamster kidney cells transfected with a p73α expression vector. (Bottom right panel) Hoechst dye stained nuclei corresponding to field presented at left.

of the transcript that could theoretically form a stem-loop structure, possibly an indication of regulatory function. Interestingly, sequencing of p73 transcripts from a wide variety of nonneuroblastoma cell lines, listed in Table 1, revealed only the G/C allele, while three of eight neuroblastoma lines possessed an A/T allele. We used PCR-restriction length analysis (PCR-RL) to screen rapidly for allele types at both the DNA and transcript levels, taking advantage of the additional Sty1 site resulting from the double substitution (G→A and C→T) in exon 2 (Figures 4b and 4c). Notably, the single remaining allele of both IMR-32 and CHP-212 bears the A/T polymorphism (Table 1). We were especially intrigued by the analysis of the SK-N-SH cell line, which shows no *p73* LOH and has both G/C and A/T alleles at the genomic level. At the transcript level, however, using RT-PCR-RL, we could only detect expression of the A/T allele (Figure 4c). This result suggested that, at least in SK-N-SH cells, p73 protein is a consequence of monoallelic expression. Another possibility that might explain this result is that our RT-PCR-RL assay may favor one allele over the other. We addressed this issue by performing the assay on G/C and A/T transcripts mixed at known ratios and showed that this assay was not obviously biased for one allele. Moreover, this analysis showed that we could detect both transcripts even when they are present in ratios of 20:1 (Figure 4d). To examine further the possibility of monoallelic expression of *p73*, we performed PCR-RL on transcripts of peripheral blood cells from five healthy donors determined to be G/C;A/T heterozygotes. All five PCR-RL assays revealed a *p73* pattern corresponding to either, but not both, the A/T or the G/C allele, supporting the notion of monoallelic expression of *p73*, at least within the 20-fold sensitivity of this assay (Figures 4c and 4d). Thus, monoallelic expression of *p73* may have particular significance for neuroblastoma and other tumors that display 1p36 LOH, as deletion of the active allele may result in a nearly complete loss of p73 activity. Finally, p73α protein expression was easily detected in a majority of nonneuroblastoma cell lines. In contrast, only two of eight neuroblastoma cell lines yielded p73α signal on Western blots, and these exclusively express the A/T transcript (Table 1).

p21^{waf} Induction and Growth Suppression by p73

The SK-N-AS cell line expresses no detectable p73 protein and negligible levels of p73 transcript and therefore represented an ideal model for testing the effect of reintroducing p73 (Baker et al., 1990). SK-N-AS cells were transfected with a plasmid expressing both the selectable marker for neomycin resistance (Neo^r) and either wild-type p73α or p73α(R292H), a mutant version homologous with p53(R273H) that is defective for DNA binding and transcriptional activation (Lin et al., 1994a; Ko and Prives, 1996). Wild-type p53 or p53(V143A) (Baker et al., 1990) were transfected separately and used for comparison with p73 samples. Cells were grown in the presence of G418, a neomycin analog, and one set of plates harvested after 48 hrs for Western blotting analysis. Lysates from the transfected cells were probed with antibodies to detect the expression of *p73* or *p53* as well as *p21*^{waf}, a known p53 target gene (El-Deiry et al., 1993). Significantly, cells expressing wild-type p73 showed elevated levels of p21 protein, comparable to those seen in wild-type p53 transfectants, whereas mutant p73- and mutant p53-expressing cells both failed to show a similar *p21*^{waf} induction (Figure 5a).

We further tested the effect of exogenously expressed p73 on SK-N-AS cells using a standard colony assay (Baker et al., 1990). Identical sets of plates to those above were maintained under G418 selection for 3 weeks and assayed for colony production. No colonies were obtained from cells expressing wild-type p73 or p53 (Figure 5b), while the DNA-binding domain mutants of each yielded high numbers of colonies. Although the physiological significance of colony assays in general is unclear, the obvious distinction between the wild-type versions of p73 and p53 and their counterpart mutants that fail to bind DNA suggests that the observed growth suppression and endogenous *p21* activation in SK-N-AS cells are a function of the transcription activity of p73 and p53.

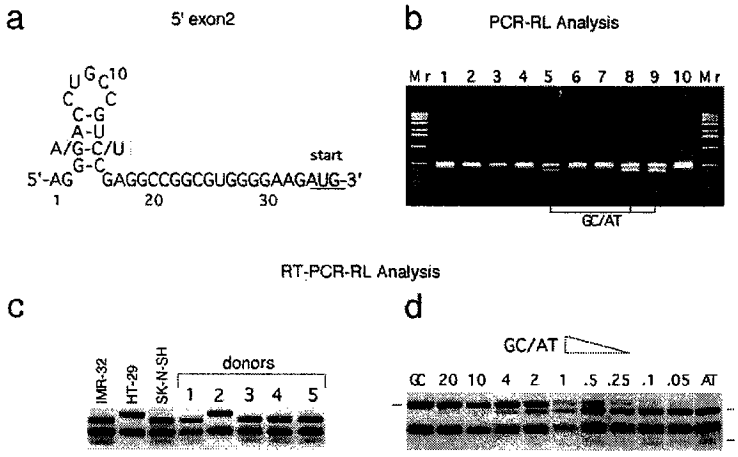


Figure 4. Monoalleleic Expression of *p73* Gene

(A) Sequence of exon 2 alleleic variants of p73α transcripts deduced from RT-PCR product sequencing. The two alleles, which represent dinucleotide substitutions at position 4 and 14 of exon 2, are G4/C14 and A4/T14 and appear at a region of the 5'-untranslated sequence that might form a stem-loop structure.

(B) Genotypic analysis of *p73* G/C and A/T alleles in ten normal blood donors using PCR-RL. Genomic PCR products that include exon 2 yield a 482 bp fragment. Sty1 digestion of PCR products yields two smaller size fragments from AT allele-derived amplicons of 376 bp and 106 bp, respectively, whereas the GC allele-derived amplicons remain uncut (lanes 1, 2, 3, 4, 6, 7, and 10). The lanes 5, 8, and 9 show heterozygotes GC/AT donors.

(C) Analysis of G/C A/T *p73* allele expression using Sty1 and Nar1 digestions of transcript RT-PCR products from cell lines and five p73 heterozygote GC/AT individuals (1–5). As with the genomic analysis, the double digestion of RT-PCR products yields specific fragments identifying each type of allele transcript-derived amplicons (284 bp for GC and 234 bp and 50 bp for AT transcripts). IMR-32, SK-N-SH, as well as blood cells of individuals 1, 3, 4, and 5 predominantly express the A/T allele at the transcript level, while HT-29 and individual 2 express the G/C allele in transcripts.

(D) A/T G/C titration assay to determine sensitivity and bias potential of RT-PCR-RL analysis for alleleic expression. G/C and A/T transcripts from total mRNA of HT-29 and IMR-32 respectively were quantified by Northern blots and mixed at the ratios indicated and analyzed by the RT-PCR-RL assay described for (C). A/T and G/C alleles can be detected even when mixed with a 20-fold excess of the other allele.

p73 Is Not Activated by Actinomycin D or UV Irradiation

In light of the structural similarities between p73 and p53, we asked whether *p73*, like *p53*, is induced by agents that activate the DNA damage checkpoint (Ko and Prives, 1996). To do this, p53 and p73 protein levels were assayed in IMR-32 cells following exposure to either actinomycin D or ultraviolet radiation (Kessis et al., 1993; Caelles et al., 1994). Actinomycin D at low concentrations (1 nM) activates the DNA damage checkpoint through producing DNA strand breaks, while at higher concentrations (1 to 2 μM) inhibits transcription (Kessis et al., 1993; Caelles et al., 1994). After 24 hrs of treatment with 1 nM actinomycin D, p53 and $p21^{waf}$ levels in the cell are markedly elevated, while p73α levels appear unaffected (Figure 6a). At micromolar concentrations of actinomycin D that inhibit transcription, p53 levels continue to rise above those of untreated cells, presumably due to a stabilization of the p53 protein. In contrast, p21 and p73α protein levels were not enhanced by actinomycin D over those of untreated cells (Figure 6a). Similar results were obtained with other cell lines.

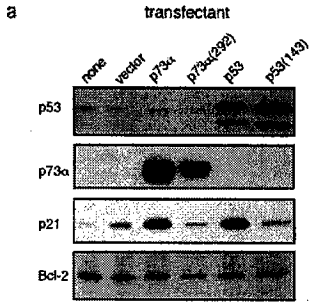
To assess further the effect of DNA damage on p73α levels, IMR-32 cells were exposed to ultraviolet (254 nm) radiation and subsequently probed with antibodies to p73α, p53, and p21. Although p53 protein was markedly increased in cells at 15 hrs after irradiation, p73α levels fail to show a similar increase after such treatments (Figure 6b). A similar failure to induce p73α was observed in cells exposed to gamma radiation or genotoxic agents such as doxorubicin (data not shown). Thus, despite the structural similarities between p73 and p53 and their common ability to induce $p21^{waf}$, these proteins do not respond in a similar manner to DNA damaging events.

Discussion

The identification of a novel gene located at chromosome 1p36.2-3 that encodes proteins with significant homology to p53 may have implications for our understanding of the etiology of neuroblastoma and other tumors as well as for *p53* evolution and function. The remarkable homology between the core domain of p73 and the DNA-binding domain of p53, together with p73's ability to induce the $p21^{waf}$ protein, suggest that p73 acts, in part, as a transcription factor. While it is not obvious that p73 and p53 share common functions, the presence of distal 1p alterations in a wide array of tumors in addition to neuroblastoma, including melanoma (Dracopoli et al., 1989), hepatocellular carcinoma (Yeh et al., 1994), and ductile breast carcinoma (Genuardi et al., 1989), supports the notion that p73 operates in pathways that coordinate cell growth, death, and differentiation.

Neuroblastoma: LOH and Monoallelelic Expression of *p73* Gene

Neuroblastoma is thought to arise from primitive neuroectodermal stem cells that fail, at various stages, to differentiate into sympathetic neurons, Schwann cells, or melanocytes (Knudson and Meadows, 1980; Ross et al., 1995). Associated cytogenetic characteristics include discrete and gross deletions of the short arm of chromosome 1, the amplification of *N-Myc*, and disturbances in cell ploidy (Takeda et al., 1994; Ambros et al., 1995; Caron et al., 1995; Cheng et al., 1995). Genetic analyses of neuroblastoma tumors harboring discrete 1p36.2-3 LOH have shown this deletion to be sustained predominantly by the maternally derived chromosome, thereby implicating one or more imprinted tumor suppressor genes in this region (Versteeg et al., 1995). In



b

transfectants	neo^r colonies
none	0
vector	550±120
p73α	0
p73α (R292H)	372±85
p53	0
p53 (V143A)	405±96

Figure 5. Expression of Exogenous p73 and p53 in SK-N-AS cells
(A) SK-N-AS cells were transfected with vectors expressing Neo^r alone or together with p73α, the p73α mutant (R292H), p53, or p53(V143A), grown for 48 hr, and analyzed by Western blot using antibodies to p53, p73, p21^{waf}, and Bcl-2.
(B) Colony formation in SK-N-AS cells transfected as in (A) after growth for 3 weeks under G418 selection.

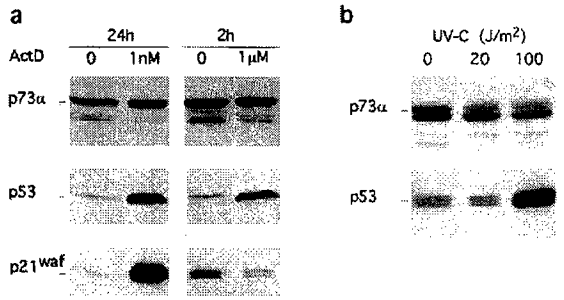


Figure 6. *p73* Induces *p21* but Is Not Responsive to DNA Damage
(A) Immunoblots of lysates prepared from IMR-32 cells treated with either 1 nM actinomycin D for 24 hrs or 1 μM actinomycin D for 2 hrs were probed with antibodies to p73α, p53, and p21^{waf}.
(B) Immunoblots of lysates prepared from IMR-32 cells grown for 15 hrs after 254 nm UV-C irradiation (20 or 100 Joules/m^2) probed with antibodies to p73α and p53.

contrast, the more aggressive stage 3 and 4 neuroblastomas appear to have amplified *N-Myc* and have sustained larger telomeric deletions, including 1p36 and 1p35 from chromosome 1 of random parental origin (Cheng et al., 1995). Whether 1p35 harbors additional tumor suppressor genes or one that affects *N-Myc* amplification or modifies expression of other genes on 1p is unknown, but it is obvious that neuroblastoma is a highly complex and heterogeneous disease involving the disruption of activities at multiple loci.

The chromosomal localization and monoallelic expression of *p73*, its frequent LOH in neuroblastoma, and its conspicuous lack of expression in a majority of neuroblastoma cell lines are all consistent with the notion that *p73* is a candidate for the putative, imprinted neuroblastoma suppressor gene at 1p36. Importantly, *p73* maps to the distal border of the consensus deletion found in neuroblastoma, as defined by a wide range of tumor cell lines (Cheng et al., 1996). It is interesting to note, however, that a constitutional 1p36 deletion in one case reportedly retained the D1Z2 marker at 1p36.33 (Biegel et al., 1993). This potentially places *p73* immediately distal to the region of overlap between the constitutional case and deletions in neuroblastoma tumor cell lines. It remains possible, nonetheless, that the constitutional deletion encompasses a region extremely close to *p73* and could mediate its disruption without a strict deletion of the gene. On the other hand, various studies have reported tumor cell lines and constitutional translocations at 1p36 whose break points do not map within the deletion in SK-N-AS. This has led to the speculation that multiple neuroblastoma suppressor genes exist at 1p36 (Takeda et al., 1994; Amler et al., 1995; Laureys et al., 1995; Versteeg et al., 1995). The disruption of *p73*, then, may be one in a number of events that contribute to the onset and progression of neuroblastoma.

While the majority of neuroblastoma cell lines analyzed here lacked *p73* expression, we were able to detect protein in two exceptions: the SK-N-SH cell line, which lacks detectable 1p or *p73* LOH, and IMR-32, which harbors a large 1p35-36 deletion characteristic of *N-myc*-amplified neuroblastomas. Notably, sequence analysis of p73 transcripts in both SK-N-SH and IMR-32 revealed a dinucleotide A/T polymorphism in the 5′ untranslated region. Using this polymorphism, we demonstrated monoallelic expression of the *p73* gene not only in cell lines but also in peripheral blood cells of normal donors. To date, we have analyzed only one informative family regarding the parental dominance in expression of *p73* alleles. In this case, a normal A/T;G/C heterozygote donor was found to express the A/T allele at the transcript level, while the maternal and paternal genotypes were A/T;A/T and G/C;G/C, respectively (data not shown). While obviously limited, this analysis shows a case in which the *p73* gene, like the putative tumor suppressor at 1p36, is expressed predominantly from the maternal allele. Further, is there any significance to the observation that in the only two neuroblastoma lines examined with p73 protein expression, namely IMR-32 and SK-N-SH, this protein was derived from an A/T transcript? At present, we have no experimental evidence for a functional difference between the G/C and A/T alleles. We note, however, that the p73 transcripts contain an in-frame CUG codon 5′ of this polymorphic region, which, at least in several genes, including *basic fibroblast growth factor* and *C-Myc*, acts as an alternative translation initiation codon (Prats et al., 1989). Moreover, conceptual translation from this CUG codon yields a coding sequence somewhat homologous to that of the transcriptional activation domain of p53. We are presently investigating the possibility that this 5′ CUG codon is in fact an alternative site for the initiation of p73 translation.

In this study, we concentrated on neuroblastoma cell lines showing 1p LOH and found that the remaining *p73*

allele lacked mutations similar to those that inactivate p53. However, as only ~30% to 40% of sporadic neuroblastoma tumors display an obvious 1p LOH (Caron et al., 1995), an extensive analysis of neuroblastoma tumors will be required to determine the actual significance of *p73* lesions, monoallelic expression, and the A/T polymorphism in the development of neuroblastoma and other diseases.

p73 Function

The identification of *p73*, its homology with *p53*, and its link to tumor suppressors at 1p36 raise fundamental questions regarding *p73* function in development and cell cycle control. Does p73, for instance, act in a manner similar to p53 to sense cellular stresses such as DNA damage and hypoxia and integrate this information for cell cycle and cell death regulation? Whereas p73 is shown to be capable of enhancing levels of endogenous $p21^{waf}$ protein, it is not induced in cell lines by agents, including UV radiation and actinomycin D, that result in p53 stabilization and activation. Although experiments are under way to determine what signals influence p73 expression, it is apparent that p73 and p53 may be serving distinct functions in the cell. An equally pressing question is whether p73 and p53 interact to yield novel activities not displayed by either molecule alone. Given the dramatic consequences of heterodimerization by members of the c-Myc family (c-Myc, Myb, Max, and Mxi1) on target gene expression (Bernards, 1995), it will be important to determine if such interactions occur amongst p53-like proteins. Our initial efforts focused on the yeast two-hybrid system for testing potential interactions between p53 and p73. Interestingly, both p53 and p73β show strong homotypic interactions, while p73α has a very low propensity for self interactions in this assay. Moreover, p73β displays an ability to interact with both p73α and p53, despite its preferential association with other p73β molecules. Although we are presently examining p73–p53 interactions in a variety of cells, the data obtained from the yeast interaction assay suggest that such interactions are possible and may be likely. The analysis of p73–p53 interactions in cells may be especially critical for understanding neuroblastoma, as p53 is often wild type in this disease, and yet recent studies indicate that it is aberrantly cytoplasmic (Moll et al., 1995). An intriguing, though speculative, explanation for this unusual behavior of p53 in neuroblastoma is that p53 requires p73 for some activities that are lost in the absence of p73. Definitive answers to whether p73 has consequential interactions with p53 will obviously require extensive genetic and biochemical studies.

Major questions remain for understanding possible p73 functions in cell cycle regulation and growth control. Curiously, the majority of nonneuroblastoma tumor cell lines was found to express high levels of wild-type p73 transcript and protein, suggesting a role for p73 in proliferation. This observation is perplexing in that most normal tissues show low levels of p73 transcript and protein (data not shown). One possible explanation for elevated p73 in tumor cell lines is that a disruption of normal *p53* function, as seen in a majority of these cell lines, results in compensatory or deleterious upregulation of *p73* expression. In this scenario, *p73* may then either assume activities of *p53* in cell growth regulation or promote survival of a disregulated cell. Importantly, recent studies have begun to establish links between active cell cycle progression and differentiation (Huttner and Brand, 1997), thereby suggesting a potential mechanism by which p73 might be required for differentiation of neuroectodermal stem cells. Analysis of p73 activity during differentiation and throughout the cell cycle should provide insight into these issues.

It is unclear at present how the identification of this novel gene will impact on our understanding of *p53*. From an evolutionary standpoint, however, it is interesting to note that *p73* shares greater homology with p53-like proteins found in mollusks than with p53 itself. It is formally possible that *p53* evolved from a more primitive, *p73*-like gene involved in many aspects of differentiation and growth control to assume more specific functions in cell cycle control and tumor suppression. It is also possible, given the general quadruplication of the *Hox* complex and other essential genes at the chordate–vertebrate transition (Holland, 1996), that other *p53*-like genes exist and comprise a p53 regulatory network.

Finally, the discovery of a gene related to *p53* will likely contribute to our understanding of the complex etiology of neuroblastoma and other diseases involving 1p LOH and may lead to new avenues in developmental and cell cycle regulation, tumor biology, and alternate strategies for cancer therapy.

Experimental Procedures

Tissue Culture

All cell lines were grown in Dulbecco's modified Eagle's medium (DMEM) containing 4 g/l glucose, 10% fetal calf serum, 2 mM glutamine, and 5000 μg/ml penicillin and streptomycin.

Two-Hybrid Assay

The two-hybrid assay was performed essentially as described (Gyuris et al., 1993). Nucleotide sequences corresponding to amino acids 72–393 of p53, 85–636 of p73α, and 85–499 of p73β were introduced into the vectors pEG202 (resulting in a fusion protein with the LexA DNA-binding domain) and pJG4-5 (resulting in a fusion protein with a transcriptional activation domain). The yeast strain EGY48 was transformed with a pEG202 plasmid, a pJG4-5 plasmid, and the pSH18.34 plasmid containing the *lacZ* gene under the control of eight lexA operators.

$p21^{waf}$ Protein Induction Assay

p73 cDNAs were cloned into a pCMV1 vector containing a 200 bp lamin 5′-untranslated region and encoding an N-terminal myc tag (Heald et al., 1993). pCMV-p53 and pCMV-p53V143A were gifts of Dr. Bert Vogelstein (Baker et al., 1990). IMR-32 cells were transfected on 100 mm plates with 20 μg of the indicated plasmid and extracts prepared by lysis in SDS sample buffer. Lysates were fractionated on a 10% polyacrylamide gel and transferred to Immobilon membranes by electrophoresis. Membranes were probed with a p21 polyclonal antibody (Calbiochem) and developed by chemiluminescence (Pierce).

DNA Damage Response

Cell lines, including IMR-32, MCF-7, and SK-N-SH, were grown to 50% confluence and incubated with either 1 nM or 1 μM actinomycin D (Calbiochem) for 24 hrs and 2 hrs, respectively. Cells were then washed with PBS, lysed in 2× SDS sample buffer, fractionated on 10% polyacrylamide gels, and electrophoretically transferred to Immobilon (Millipore) membranes. Membranes were probed with a polyclonal antibody to p73α, a monoclonal antibody to p53 (ATCC,

Bethesda), or a monoclonal antibody to p21 (Calbiochem). For examining responses to ultraviolet radiation, media was removed from plates and cells exposed to 20 and 100 Joules/m^2 254 nm UV-C. Media was returned to the plates and cells grown for an additional 15 hrs prior to lysis and Western blotting, as above.

Fluorescence in Situ Hybridization (FISH)

The *p73* cosmid probe was labeled by nick translation using biotin-16-dUTP (Boehringer Mannheim) according to a BRL (Bethesda Research Laboratories) protocol. Twenty microliters of the hybridization solution containing 40 ng of biotinylated probe and 10 μg of human placental DNA was incubated at 80°C for 5 min. DNA was allowed to reanneal at 37°C for 6 hrs before placing on the slides. Chromosome spreads of peripheral blood lymphocytes and the SK-N-AS, SK-N-MC, and IMR-32 neuroblastoma cells were prepared from asynchronous populations grown in DMEM with 10% fetal bovine serum and exposed to colcemid (500 nM) or nocodazole (100 nM) for 2 hr. After hybridization in 50% formamide, 2× SSC, and 10% dextran sulfate for 12 hr at 37°C, biotinylated probes (*p73*, D1Z5) were detected with an FITC-conjugated anti-biotin antibody (GIBCO), and the digoxigenin-labeled probe (D1Z5) was revealed by a rhodamine-conjugated anti-digoxigenin antibody (Boehringer Mannheim). Chromosomes were counterstained with either DAPI (4,5-diamino-2-phenylindole) or propidium iodide, and slides were mounted in antifade solution (Vectastain).

Transfections and Immunofluorescence

Construction of the myc-tagged p73 vectors, transfection, and immunofluorescence were done essentially as described (Heald et al., 1993). Cells were fixed with 3% formaldehyde in phosphate-buffered saline (PBS), blocked with 3% milk in PBS containing 0.1% Triton X-100 (PBST), and sequentially incubated with primary and CY3-conjugated secondary antibodies (Jackson ImmunoResearch) for 30 min each at room temperature. DNA was labeled using Hoechst dye 33258 (Sigma) at 1 μg/ml for 1 min. The 9E10 mouse monoclonal antibody against the c-Myc epitope was obtained from the Cell Culture Facility at Harvard University. The rabbit polyclonal anti-p73α antibody was generated against a C-terminal p73α (427–636) glutathione S-transferase fusion protein.

SK-N-AS Colony Formation Assay

SK-N-AS cells on 100 mm plates were transfected with the indicated pCDNA3 vectors using the calcium phosphate method. Forty-eight hours later, cell extracts were prepared, immunoblotted, and probed with antibodies to p73, p53, p21, and Bcl-2. Identical sets of plates were grown in 500 μg/ml G418 (GIBCO) for 3 weeks, fixed, stained, and counted (Baker et al., 1990).

Molecular Biology Methods

RNA preparation, Northern blot analysis, immunoblotting, genomic and cDNA cloning, as well as screening and nucleotide sequencing, were performed using standard protocols (Maniatis et al., 1992).

***p73* Gene and Transcript Analysis**

cDNA synthesis was performed using 5 μg of total RNA incubated in a 20 ml volume reaction containing 50 mM Tris-HCl [pH 8.3], 10 mM DTT, 10 mM KCl, 0.5 mM dNTP, 30 μg RNAsin (Promega), 150 μg superscript II reverse transcriptase (GIBCO; BRL) for 1 hr at 37°C. PCR reactions were performed with either 2 ml of reverse transcriptase products or 300 ng genomic DNA in 50 ml reaction containing 50 mM Tris-HCl [pH 9.2], 16 mM $(NH_4)_2SO_4$, 1.75 mM $MgCl_2$, 10% DMSO, 0.3 mM each primer, and 3.5 μg of TAQ and PWO DNA polymerases (Boehringer Mannheim). The amplification sequence consisted of 30 cycles of 95°C/0.5 min, 58°C/1 min, 68°C/2.5 min after starting with a denaturation step at 95°C for 1 min and ending at 68°C/10 min. The amplicons were purified by spin dialysis sequentially on S400 and P10 resins. For restriction length analysis on transcripts, amplicons were generated as described above using primers (5′CGGGACGGACGCCGATG and 5′AGACCGTAGACCGTC ATC derived from the p73 cDNA sequence and analysed on 1.5% agarose gels after digestion with Nar1 and Sty1 restriction endonucleases. Restriction length analysis for genomic DNA was performed using two sequential, nested PCR reactions. For PCR-1, primers 5′CACCTGCTCCAGGGATGC and 5′AAAATAGAAGCGTCAGTC derived from intronic sequences were employed. For PCR-2, 2 ml of purified PCR-1 reaction products were used with a more internal set of primers (5′CAGGCCCACTTGCCTGCC and 5′CTGTCCCCAAG CTGATGA). The resulting amplicons were Sty1 digested and analysed on 1.5% agarose gels.

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EMBL Accession Number

DNA sequences corresponding to the human p73α and p73β cDNAs have been deposited in the EMBL database under the accession number Y11416 EMBL.